U. S. National Institute of Arthritis
and Metabolic Diseases. Laboratory
Research.
Analysis of NIH program activities.

To the Scientific Staff, NIAMD

The accompanying summaries, which constitute a part of the "Analysis of NIH Program Activities, 1956" have been prepared by Dr. Bunim and myself, based largely upon the project descriptions which you have supplied. The purpose of distributing this material to you is to give you the opportunity to learn of activities within our Institute which may not be familiar to you. In the event that you discover herein some unfamiliar activity the source of which you are unable to identify, if you desire more information, Dr. Bunim's office can supply the source in the clinical area and I shall be happy to do the same for the basic sciences. It is our hope, by this device, to reduce the barriers to transmission of scientific information which may now exist.

We of the clerical staff are grateful to the scientists for their cooperation in the preparation of this report and for the performance of the experiments upon which it is based. We apologize in advance for any errors which may have crept into the preparation of these summaries.

With Seasons Greetings!

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Analysis of NIH Program Activities

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U.S National Institute of Arthritis and Metabolic Diseases

Laboratory Research

Introductory Comments - The comprehension of biological phenomena results from the fusion of information derived from many disciplines. The normal operation of the organism may be studied in the intact animal, the isolated intact organ, the sliced or homogenized organ, the individual cell, the sub-cellular particle, the soluble multi-enzyme system or the pure individual enzyme. The chemical composition of the substrate, the enzyme which catalyzes, the hormone which regulates and the fluid which contains the reaction must all be investigated. To gain further insight, the biological system may be insulted, and the insult may be an alteration of diet, the administration of a foreign agent, whether microbial or chemical, the application of some form of energy or other physical stress, or the alteration of normal anatomy by surgery.

Examples of all of these and other approaches will be found in the ensuing pages. One may ask: Which of these approaches is the best? The answer quite clearly is that no one approach is superior to all others. At a particular time in history, the answer to a particular problem will appear to a particular scientist to be most accessible by a particular approach. This however is illusory since each scientist necessarily has certain techniques at his command in which he has reliance and skill. Each succeeding year presents us with new additional techniques, which, as they are mastered, make older experiments seem inexact and less than rigorous. The recurrent experience has been that for a biological truth to be securely established, compatible evidence must be obtained from biological preparations at various levels of organization examined by various techniques. This of course takes time and it is a rare event in the history of science to see such a truth established within one calendar year.

An example of the effectiveness of the multidisciplined approach will be found below in the report of the origins and fates of the formiminoamino acids. The very satisfactory progress which we are reporting in this area of biochemistry was made possible because the techniques of organic synthesis, chemical isolation and identification, enzyme purification, chemical and microbiological analysis, spectrophotometry and nutrition research were applied to intact animals, to tissues and enzymes derived from these, and to microorganisms.

A review which covers the events of a single calendar year, as the present one does, is an artificial slice of history, resembling, in some regards, a single frame in a motion picture film. Its significance cannot

be interpreted without a perspective of the past and a vision of the future. The analysis of scientific experiments over such a brief span is feasible, but, with rare exceptions, the synthesis of scientific findings into scientific truths requires the consideration of a far longer segment of time.

Carbohydrate Chemistry and Metabolism - The chemical structures and reactions of the sugars continue to be the keystone to the understanding of the metabolism of these compounds. A number of significant advances may be recorded at an organic chemical level. A novel intramolecular migration from position 1 to position 2 of a substituent benzoyl group on ribose has been observed, and the existence of cyclic ortho acid esters of sugars has largely been discredited. A new class of anhydrosugars has been discovered, represented by 1,5-anhydro-β-D-ribofuranose which is at once both a furanose and a pyranose. The structure of the intensely sweet natural product, stevioside, has been elucidated. It is a glycoside wherein two glucose residues are in β(1⟶2) linkage. It is thus a sophoroside, and except for sophorose itself, the only known member of this class. The conditions determining yields of the sugar anhydrides of many hexoses and heptuloses have been studied.

The central role of xylulose 5-phosphate in the oxidative metabolism of glucose has been established. This pentose has been shown to be both a substrate for transketolase and also the initial pentose formed by this enzyme. Transketolase, which effects transfer from one sugar residue to another of a two-carbon (active glycolaldehyde) residue, shows an absolute requirement for the trans-configuration at C-3, C-4 of reactive ketoses. A bacterial fermentation of xylulose 5-phosphate has been found to require inorganic phosphate and to yield acetyl phosphate. Active acetyl is thus produced without intervention of coenzyme A. In this "phosphoketolase" reaction, as in the transketolase reaction, a requirement for thiamine pyrophosphate was shown. Since the discovery by NIAMD scientists that ribulose 1,5-diphosphate was the unique primary acceptor of carbon dioxide in photosynthesis, the reactions of this sugar have been a matter of continued interest. A phosphatase has been isolated from spinach which preferentially removes one of the two phosphate groups. This is being further studied in the hope of gaining access to the presently unavailable ribulose 5-phosphate. The reaction whereby another pentose, xylulose, enters mammalian biochemistry, has been clarified by the discovery of a kinase in liver which catalyzes the formation of xylulose phosphate. This finding has bearing upon the ultimate understanding of the human congenital defect, pentosuria.

The metabolism of a number of sugar acids has been studied. Galacturonic acid has been found to disappear when incubated with rat liver extract, yielding products presumed to be on the pathway of ascorbic acid synthesis. In contrast to findings of others, glucuronic acid is found to be abundantly catabolized by the intact animal. New analytical methods which discriminate between glucuronic acid and glucosiduronic acids have been devised in relation to this study. The initial step in the metabolism of gluconic acid has been identified as conversion to 6-phosphogluconic acid. The enzyme which catalyzes this reaction has been purified, its specificity and cofactor requirements have been identified. By this reaction gluconic acid enters into well known metabolic channels.

The application of the isotope technique to certain problems of mechanism of enzyme-catalyzed reactions has been very rewarding. The three hexoses, glucose 6-phosphate, fructose 6-phosphate and mannose 6-phosphate are interconvertible in the presence of appropriate mammalian isomerases. Fructose 6-phosphate, the ketohexose, occupies an obligatory intermediate position between the two aldohexoses. Of the two hydrogen atoms bound to carbon-1 of fructose, it has been shown by use of hydrogen isotopes that one is related uniquely to the glucose transformation while the other one is peculiarly labilized in the mannose transformation. Similarly, in two reactions of dihydroxyacetone phosphate studied one of the two hydrogen atoms on carbon-3 is labilized by aldolase, the other by phosphotriose isomerase.

The metabolism of the important sugar galactose, has been the subject of intensive study. Virtually all of the reactions whereby galactose enters metabolic pathways have been delineated. These involve phosphorylation by a specific kinase, interaction of this product in the presence of a transferring enzyme with uridine diphosphate glucose to yield uridine diphosphate galactose, followed by epimerization of the latter compound about carbon-4 to yield again the glucose derivative. This latter epimerization has been shown to require diphosphopyridine nucleotide and is therefore believed to proceed via two oxidoreductive steps. Of particular interest are studies of the metabolic defect in the congenital disease, galactosemia. In this condition, an inborn intolerance toward galactose, galactose 1-phosphate accumulates in the tissues where it acts deleteriously. The ultimate injuries include cataract, mental deficiency, hepatomegaly and other manifestations. Assay for the several enzymes possibly involved has revealed the complete absence from erythrocytes and from liver of the transferring enzyme and normal abundances of all other components. Since the manifestations of the disease are probably preventable by elimination of dietary galactose, early diagnosis is important. In view of the now established "molecular" nature of the disease and its attribution to a single enzyme defect, such early diagnosis by enzymologic assay becomes feasible. The discovery that this and several other congenital defects may result from the lack

of a single enzyme provides a useful "marker" in human and animal genetic studies which are being initiated. Of particular interest are attempts to demonstrate, in the spermatozoa of affected males, evidences of the enzyme deficiency which is present and demonstrable in somatic cells.

Whereas diabetes (to be considered subsequently) is the most frequent important disturbance of carbohydrate metabolism, others, like galactosemia, do occur and their study is certainly rewarding. Another such congenital defect is pentosuria, in which disease the sugar L-xylulose appears in the urine. Recent studies employing chromatographic techniques have shown that xylulose, contrary to earlier reports, is a normal though minor urinary constituent and indeed is but one of several sugars found in small amount in normal human urine.

The metabolism of polysaccharides of high molecular weight has also been the subject of study. The detailed nature of the turnover of glycogen in muscle and in liver of intact animals has been clarified. It has previously been reported that glycogen is metabolically inhomogeneous at an intramolecular level. More recent studies reveal that this inhomogeneity is also extended to the intermolecular level. In liver it is the smallest glycogen molecules, in muscle it is the largest molecules that are metabolically most active. The characteristics of structure of glycogen and of enzyme activity in these two tissues have been studied in vitro in an attempt to learn the basis for the in vivo differences observed. Another polysaccharide, chondroitin sulfuric acid, has also been studied. This material conjugated to protein, a major component of the extracellular matrix of cartilage, hence of interest in arthritis research, can be extensively degraded in vivo by some as yet unidentified constituent of crude papaya latex. A product resembling chondroitin sulfate histologically can be observed to enter the blood stream after papain injection while the several cartilages of the body are losing rididity and staining capacity. The nature of the enzyme in crude papain responsible for this effect and the identification of the products of its reaction are subjects of present investigation.

The microbial fermentations of oxaloacetic acid have been studied. An enzyme, oxaloacetic hydrolase has been secured from an enrichment culture of Aspergillus niger. Oxalic and acetic acid were identified as reaction products. The further decomposition of oxalic acid may be effected by a second new enzyme, oxalic decarboxylase, which has been extracted and extensively purified from Collyvia veltipes.

Amino Acids, Amines, Purines and Nucleic Acids - A coordinated and frequently collaborative advance may be recorded in the chemistry and biochemistry of the nitrogenous constituents of living cells. Especial interest attaches to hydroxy-derivatives of amino acids and amines. 5-hydroxy-DL-tryptophan has for the first time been resolved and all the pharmacologic activity has been found to reside in

the L-antipode. The same material was a potent inhibitor of insulinase but was no more effective in this regard than was the simpler 5-hydroxyindole. Other novel indole derivatives which have been identified and synthesized are a product derived biologically from lysergic diethyl amide and another material isolated from Claviceps purpurea. The application of color reactions of indoles to histological preparations has yielded some important information. Of particular interest was the observation of an indole type of material in the α-cells of the islets of Langerhans, which generate glucagon, a tryptophan-containing protein. No similarly staining material could be demonstrated in the β-cells which generate insulin, a protein devoid of tryptophan. Definitive configurational assignments have been made to a number of hydroxyamino acids. These include the naturally occurring homolog of hydroxyproline, 5-hydroxypipecolic acid and δ-hydroxylysine. Hydroxyproline and hydroxylysine, it will be recalled are found in nature chiefly in collagen, the characteristic extracellular protein of connective tissue. A large number of derivatives of hydroxyproline have been synthesized and certain of these, when tested in a hydroxyproline-producing carrot preparation, were found to exhibit striking antimetabolic activity. Various modifications and derivatives of the tyrosine and thyroxin molecules have also been prepared. Nitro-derivatives of the unnatural meta-tyrosine have been synthesized and characterized and a series of m-diphenyl ethers related to thyroxin have also been prepared. These compounds should prove useful in future studies of thyroid function.

The polyamines, spermine and spermidine, occur abundantly and are widely distributed in nature. Although found in significant amounts in mammalian tissues, these substances are surprisingly toxic. Spermine, injected into a renal artery, produces a cortical tubular degeneration, followed by arterial intimal proliferation and renal atrophy. In the hope that knowledge of their metabolism would clarify their toxicology, isotopically labeled spermine and spermidine have been prepared and their metabolic fates studied. Liver homogenates transform spermine into spermidine and putrescine. Doubly labeled (C^{14}, N^{15}) putrescine was incorporated biologically into spermine and spermidine, the three-carbon side chains arising chiefly from methionine. Perhaps due to its basic nature, spermine antagonized the anticoagulant action of heparin, which is of course strongly acidic.

Clinical disturbances in amino acid metabolism have been found in relation to the aromatic amino acids phenylalanine, tyrosine and tryptophan more frequently than with other amino acids. For example, a specific disturbance in tryptophan metabolism in tissues of the diabetic animal will be discussed subsequently. For this reason among others, considerable interest attaches to the detailed knowledge of the metabolic pathways of these amino acids. The discovery of a new class of enzymes, "oxygenases", which catalyze the aerobic oxidation of various cyclic amino acid derivatives is of importance. Studies with O^{18} revealed that the oxygen incorporated in the hydroxyl group, in each case, arises

from gaseous O_2, not from H_2O. The members of this class studied to date include tryptophan oxygenase, 3-hydroxyanthranilic acid oxygenase, aromatic hydroxylase and imidazoleacetic acid oxidase. These enzymes are formed adaptively by microorganisms and are of especial concern because they activate, by means as yet unknown, molecular oxygen. Rat liver mitochondria contain an enzyme, now solubilized, which hydroxylates kynurenine in the 3-position. A pyridoxal-requiring transaminase has been purified from Neurospora which transforms kynurenine and 3-hydroxykynurenine into kynurenic and xanthurenic acids respectively.

The transformation of aspartic acid into homoserine occurring in yeast has been studied in detail and has been dissected into three successive steps:

Aspartic acid ⟶ β-aspartyl phosphate ⟶ aspartic-β-semialdehyde ⟶ homoserine

The biological migrations of methyl mercaptan have been explored and a number of new compounds identified. Reacting with 3-phosphoglyceric acid, it yields a thiomethyl ester which is enzymatically reduced to a glyceryl thiomethyl ether. Reacting with serine, methyl mercaptan yields S-methyl cysteine. Among other products formed in yeast is one tentatively identified as L,L-β-methyl lanthionine.

Histidine, and its decarboxylation product, histamine, continue to be subjects of biochemical study. It had previously been shown that formiminoglutamic acid arises directly from histidine. The next lower homolog formiminoaspartic acid has now been identified as a product of histamine metabolism via the steps:

Histamine ⟶ imidazoleacetic acid ⟶ formiminoaspartic acid ⟶ (in Pseudomonas) formylaspartic acid

The further observation that formiminoglycine is a normal bacterial product of purine catabolism extends to three the list of amino acids known to occur in living cells as their formimino derivatives. It therefore becomes of pressing interest to learn of the biological fate of the formimino group. Fortunately scientists working collaboratively in several laboratories of NIAMD have been able to supply many of the needed answers. These studies have been carried out with both the glycine and the glutamic acid derivatives and complete parallelism has thus far been encountered. The formimino group has been shown to migrate intact to tetrahydrofolic acid, a member of the vitamin B group. Here it is decomposed stepwise, yielding successively ammonia and formic acid,

over the following steps:

1. Formiminoglycine + tetrahydrofolic acid → N^5-formiminotetrahydrofolic acid + glycine.
2. 5-formiminotetrahydrofolic acid → 5,10-methenyltetrahydrofolic acid + ammonia.
3. 5,10-methenyltetrahydrofolic acid → N^{10}-formyltetrahydrofolic acid.
4. N^{10}-formyltetrahydrofolic acid + ADP + P_i → ATP + formic acid + tetrahydrofolic acid.

Attention should be directed to the previously unknown intramolecular transfer of a substituent on folic acid from N^5 to N^{10}. Of particular interest is the novel "substrate phosphorylation" occurring in the 4th step. This is also revealed when the sum of the preceding four reactions is considered;

Formiminoglycine + ADP + P_i → ATP + glycine + formic acid + ammonia

By virtue of this phosphorylation additional energy for useful work is made available to the cell from the catabolism of histidine, histamine, xanthine and possibly other precursors.

The pathway of histidine biosynthesis appears to be notably different from that of histidine catabolism. Five steps, commencing with imidazoleglycerol phosphate, have been identified, to wit:

Imidazoleglycerol phosphate → imidazoleacetol phosphate → histidinol phosphate histidinol → histidinal → histidine

The individual enzymes catalyzing these steps and their cofactor requirements are currently being studied.

Probably the most important advance in the understanding of nucleic acid metabolism in the past year has been the development of information about polynucleotide phosphorylase. This bacterial enzyme catalyzes the reversible reaction;

Nucleoside 5'-diphosphate ⇋ polynucleotide + P_i

In the development of this information NIAMD scientists have collaborated closely with scientists at New York University. The new synthetic polynucleotides made available by this reaction have been tested as substrates for known phosphodiesterases and nucleases. Pancreatic ribonuclease has been intensively investigated in regard to its structural substrate requirements. With isotopic tracers and lytic enzymes of known specificity it has been possible to learn a great deal about the mechanism of the nucleotide phosphorylase reaction. The first evidence that this enzyme may act in a degradative sense on natural nucleic acids was secured by NIAMD scientists. Likewise the first

evidence for the existence of a mammalian nucleotide phosphorylase was secured in this laboratory.

The availability of various lytic enzymes of known specificities permits the hope that systematic sequence studies for purines and pyrimidines in nucleic acid chains may be achieved. Analogous information about amino acid sequences in proteins is of course becoming increasingly available. Already the first step, the characterization of end-groups of polynucleotides, has been achieved by NIAMD investigators who now have methods to distinguish between "5'-ended" and "3'-ended" polynucleotides.

Nutrition and Vitamins - Nutritional studies in recent years have been concerned largely with the roles of micronutrients. The organic micronutrients, vitamins, have, in most cases, been found to contribute to the synthesis of specific cofactors required in the operation of certain enzyme-catalyzed reactions. The nature of this participation, the biosynthesis of the vitamins themselves, the reasons for impeded biosynthesis in those species where dietary indispensibility is manifest, the mechanisms of degradation of vitamins, have all been areas of active research.

The folic acid molecule occurs in several modifications in nature. Thus in mammalian liver there have been found two distinct precursors of folic acid, prefolic A and prefolic B, which, though inactive in microbial assays, are enzymatically transformed into materials which exhibit folic acid activity. Incubation of folic acid or of citrovorum factor, its tetrahydroformyl derivative, with liver extracts results in loss of growth-promoting activity. In each case an aerobic rupture of the bond between the pterin and p-aminobenzoic acid appears to occur. From bacteria enzymes have been isolated which cleave N-aryl (or N-acyl)-L-glutamic acid derivatives, folic acid among others. Another bacterial enzyme has been found which hydrolytically removes the 2-amino group from the pterin of folic acid. The role of folic acid derivatives in metabolism of formimino derivatives of amino acids has been discussed elsewhere in this report. An important lead was supplied by the identification of formiminoglutamic acid, a histidine metabolite, in the urine of folic acid deficient animals. It has now been shown that histidine, as well as tryptophan, threonine and certain nucleic acid derivatives partially counteract the effects of folic acid deficiency.

Several steps in the biosynthesis of nicotinic acid from tryptophan have been further investigated. The oxidations of tryptophan and of 3-hydroxyanthranilic acid are catalyzed by enzymes of the newly defined "oxygenase" group. The enzyme picolinic carboxylase and the mechanism of its reaction have been studied. The fact that its activity in liver is greatly increased in diabetes was discovered and explored (cf. Diabetes, below). Niacin deficiency in dogs leads to black-tongue,

a disease studied by Goldberger. The diets he employed were rich in corn. Based upon human nutrition studies, experiments have been undertaken to ascertain whether treatment of corn with alkali renders it a better source of niacin. Preliminary results indicate this to be the case.

When maintained on vitamin E-free torula yeast diets, rats develop a fatal hepatic necrosis which may be prevented by an as yet unidentified nutritional accessory factor, termed Factor 3. Purification from kidney powder of Factor 3 has been under way for a few years at NIAMD, and progress is recorded. Two materials of differing properties, both exhibiting Factor 3 activity, have been separated and considerable concentration of one of these, α-Factor 3, has been effected by chromatographic and partition procedures. This product is strongly anionic, water-soluble, not extractable with phenol. Factor 3 deficiency has now been shown to lead to necrotic lesions not only in liver, but also in kidneys and skeletal and cardiac muscle, together with pancreatic atrophy. The role of vitamin E in the prevention of the hepatic necrosis that responds to Factor 3 has also been studied. Fourteen synthetic antioxidants have been compared with tocopherol both in vivo and in vitro and certain of these, notably N,N'-diphenylphenylene diamine were highly effective as prophylactic agents. Because of the finding that the synthetic antioxidants were active in the in vitro test, where vitamin E was ineffective, it has been suggested that it is some metabolic product of vitamin E which is the effective agent in vivo. This suspicion has been confirmed by the finding that two recently isolated metabolic derivatives of vitamin E were effective in vitro.

Whereas most laboratory nutrition studies have of recent years been conducted upon rats, it is obviously of interest to secure comparative data in a variety of other species. Such studies have been undertaken in the chick, mouse and guinea pig. Among the recorded findings from this study is the detection of an unidentified thermolabile growth factor, the absence of a thioctic acid requirement, and the probable absence of a requirement for vitamin E if no dietary fat is allowed, all in the chick. An exudative diathesis could be produced in the chick when vitamin E and Factor 3 were both withheld. In this species a severe vitamin B_{12} deficiency has been produced in four weeks on a purified diet. Various analogs of this vitamin have been assayed in the deficient chick. The 5-hydroxy analog was about one tenth as potent as vitamin B_{12} itself while the nitrato-, sulfato-, chloro- and acetato-cobalamins were fully active. In the chick a relative choline deficiency could be produced by administration of a competitive inhibitor, 2-amino-2-methylpropanol-1. The absence of diet fat was necessary to produce fatty liver in choline deficiency in this species. Folic acid deficiency can be produced in young mice by dietary means alone. Such mice have been found to fail to support the reproduction of lymphocytic choriomeningitis virus. The virus will reproduce satisfactorily if folic acid

is added to the diet. Growth data in the guinea pig suggest that the conversion of tryptophan into niacin occurs in this species, as in many others. A vitamin B_{12} requirement for optimal growth is present in this species. Whereas inositol is not needed on a 30% casein diet, on purified amino acids a definite requirement for inositol is shown by the guinea pig.

The rapid destruction of vitamin A injected intravenously into the rat can be now attributed to an activity in the blood, especially in the stroma of the erythrocytes. This thermolabile, presumably enzymatic activity is not inhibited by azide or by cyanide. Chick blood, in contrast to rat blood, was almost ineffective in destroying vitamin A *in vitro*.

The study of the production of obesity in rats by dietary means has been continued. Such obesity is secured on diets containing 40% of fat supplemented with methionine and aureomycin. Pathological studies have revealed an osteoarthritic type of joint lesion. Organ weights were normal except for the adrenals which weigh approximately 50% more in obese than in lean animals. Histological changes were also reported both in the adrenal and thyroid glands. Blood pressures in obese animals were normal. Weight reduction studies on obese animals revealed lowest mortalities when the initial high-fat diet was offered in limited amount.

Many nutritional studies depend upon ultramicroanalytical procedures for nutrients or their derivatives and these often are conducted by microbial methods. A laboratory is operated to carry out such services for other investigators, and a number of useful collaborations have been undertaken in this area. For example, in studies of the effects of ethylene oxide upon the diet (see below), in studies of the effect of diabetes upon the tolerance to various B vitamins, microbiological assays were essential. Among the improved methods introduced is the analysis of 5- and 10-formyl derivatives of tetrahydrofolic acid in millimicrogram amounts when in the presence of each other.

Endocrines and Endocrine Diseases - The general problems of endocrinology include isolation identification, purification, proof of structure and ultimate synthesis of the products of endocrine glands; development of analytical methods for separation and quantitation of such products; the analysis of the nature and mode of action of the hormone upon the target organ; the metabolism of the hormone; and the unravelling of the complex interactions of the several endocrine glands and associated nerve structures upon each other. It is upon the answers to these problems that understanding, diagnosis, treatment and prophylaxis of the endocrine diseases should rest. In all these areas NIAMD scientists have great interest.

Diabetes is the most frequent as well as the most important recognized endocrine disease. Because little is known of the ultimate metabolism of insulin, because the hypoinsulinism in this state may be

relative or absolute, because insulin antagonists (see below) are known to occur in the bloodstream, the development of a method for insulin assay sensitive in the range of physiological concentrations is a problem of immediate importance. The development of such a method, capable of detecting as little as $2x10^{-12}$ moles of insulin is under study. The method depends upon hypoglycemic effect of insulin upon the very sensitive diabetic hypophysectomized mouse. Even with this remarkable sensitivity, virtually no insulin was detectable in the blood of mice. Pancreatectomy leads to diabetes of varying severity in various animal species. Total pancreatectomy has not been possible in the past in common laboratory rodents because of the diffusely scattered arrangement of the gland. A very severe diabetes has now been produced in the rat by virtually total extirpation of the pancreas. Subsequent hypophysectomy in such diabetic rats results in a marked decrease in insulin requirement and an improved survival on fasting. The report of others that insulin had specific growth-hormone-like functions has been scrutinized. It was found, under carefully controlled conditions, that the growth-stimulant action of insulin in the hypophysectomized rat was entirely attributable to the increase in food intake in response to insulin.

The earliest antagonist of insulin to attract attention arises in the hypophysis (Houssay) and is now believed to be identical with or related to growth hormone. Other agents which have been shown to antagonize, directly or indirectly certain manifestations of insulin include the glucocorticoids, glucagon, insulinase and specific antibodies to heterologous insulin. An antagonist identical with none of these has been found in high frequency in the serum of patients in diabetic ketosis. This agent, in the α-globulin fraction of plasma proteins, disappears in a few hours after therapy against ketosis is established. Its chemical and physical properties, as well as its mode of action are being determined. In order to evaluate its role in the causation of diabetes, it is soon to be tested against human insulin which is being prepared by NIAMD staff. The well-known increase in tolerance toward insulin in the diabetic when in keto-acidosis is in large measure explained by the present findings.

The failure of dietary tryptophan to provide niacin in the diabetic rat has previously been reported. Attempts to reproduce this defect in diabetic dogs or humans have not been successful thus far. The defect in the liver of the diabetic rat has been studied in detail. The most striking related enzymological change is an increase, in diabetic liver, in the activity of picolinic carboxylase. This change in diabetes develops gradually after removal of the pancreas, and requires, for its development, the presence of adrenal glands.

Insulin is known to act primarily in facilitation of glucose utilization by certain tissues, especially skeletal muscle. Two new factors are under study which appear to influence this phenomenon. The first of these was encountered in the course of study of Factor 3 deficient rats when it was noted that these animals had an impaired

glucose tolerance. The Glucose Tolerance Factor (GTF), as it has been named, is not identical with Factor 3. It is present in kidney and in brewer's yeast. It is not identical with any known nutrient. Various diets have been constructed which are not necrogenic yet which lack GTF. Its isolation is being pursued. A second factor was encountered when it was noted that peripheral removal of glucose was lowered in the absence of the liver. Suitable liver extracts, when administered, were found partially to restore this rate toward normal. This material appears to be related to GTF since the extracts of livers of animals whose diets were deficient in GTF had only one quarter of normal activity in restoring the glucose extraction rate of the hepatectomized rat. Gross chemical and physical properties of this liver substance have been determined and its further study is being pursued.

A number of studies relate to the hormones of the pituitary gland. A transplantable mouse pituitary tumor which generates large amounts of adrenocorticotrophic hormone (ACTH) has been maintained and studied. The ACTH derived from it has been found to contain melanophore stimulating hormone (MSH) activity also. Furthermore, treatment of this ACTH with alkali increased its MSH activity. This finding, considered together with others, suggests that MSH may be a product of partial degradation of ACTH. By application of new chromatographic methods, thyroid stimulating hormone (TSH) has been purified three hundred fold from beef pituitary glands. By the application of this novel method of isolation to the mouse pituitary tumors, TSH has been identified in this material. Physical and chemical studies designed to yield structural information have been undertaken on TSH preparations. The newly developed assay method for TSH has been applied to human blood samples, both normal and diseased. Whereas the human blood levels were either very low or undetectable, high concentrations of TSH were found in the blood of certain mice bearing pituitary tumors.

There has long been a suspicion that the pituitary gland, several of the functions of which are to activate other endocrine organs, is itself under the directed influence of the hypothalamic nuclei. The relations of neural and humoral influences, particularly in regard to the question of the control of pituitary functions by nervous impulses, has been the subject of a sizable and continuing project. The general procedure has been to divide the brain-stem of dogs at predetermined levels and to study, directly or indirectly, residual pituitary function. As an example of the types of findings being recorded, it has been noted that dogs, months after midbrain transection, fail to respond to surgical and other stresses, as normal dogs do, with an outpouring of adrenocortical steroids. That the animals' adrenal cortices are still responsive is readily demonstrable by the injection of exogenous ACTH, which is followed by the expected rise in steroid excretion. Whereas the midbrain-transected dog appears unable to respond to stress by endogenous ACTH release, in some cases he apparently generates normal amounts of thyrotrophic hormone, as determined by measurements of depletion of thyroidal I^{131}. Various changes in

circulatory and renal functions have been noted to follow midbrain transection, evidenced by reduction of glomerular filtration, renal plasma flow and cardiac output. A marked increase in hemodynamic resistance in the kidney and in the periphery suggest that a generalized arteriolar spasm follows midbrain transection. The mechanism of an anomalous creatinuria which follows such surgery is being explored.

Steroids, which include hormones of the adrenal cortex, testis and ovary, as well as many other biologically important compounds, have been the subject of several studies. The application of modern methods of separation has permitted analyses of the complex mixtures of steroids in urine in health and disease. Among the abnormal states studied are pregnancy, Cushing's syndrome, Addison's disease, congestive heart failure, hepatic cirrhosis; also patients under treatment with ACTH, adrenocorticoids and synthetic analogs. The interconvertibility of Metacortin and Metacortalone in man was thus demonstrated. Similar urinary analyses have been conducted upon dogs subjected to midbrain transection. The fecal excretion of steroids has been explored and a 17-ketosteroid has for the first time been demonstrated in this material. Structural chemical studies on various steroids have revealed: A possible novel method of oxidation at carbon-11 of dehydroergosterol; the occurrence in animal bile of sapogenin-like compounds, of a type formerly known only in plants; the previously unknown isomerism about carbon-22 in the sapogenin side-chain; and final clarification of the structures of tomatidine and solasodine. Earlier mention was made of the natural sweetening agent, stevioside. In addition to elucidation of its carbohydrate moiety (q.v.), its aglycone, a steroid relative has also been studied and modifications of its structure have been proposed. Synthesis of amides of colchicine, compounds of therapeutic interest, has been studied.

Analgesic Drugs - The quest for analgesic drugs which might be less prone to cause undesirable side effects than drugs presently available has been pursued at an organic chemical and at a pharmacological level. Screening and assay methods for new agents have in some cases included clinical studies using a double-blind technique on patients supplied largely by NCI. A new class of compounds, in which the N-methyl of morphine was replaced by a 2-phenylethyl group has been described. When certain morphine analogs were thus altered, an increase of 50- to 100-fold in activity was found on animal test. High activity and relatively low toxicity were also found for 2'-hydroxy-2,5,9-trimethyl-6:7-benzmorphan, a compound newly synthesized by NIAMD scientists. 5-(m-hydroxyphenyl)-2-methylmorphan, while as active as morphine, has less addiction potential, at least in monkeys.

A large number of derivatives of morphine analogs have been prepared and new synthetic routes have been devised. These have included enlargement of the N-containing ring of a codeine derivative, preparation

of phenoxyacetates of the morphine series, introduction of additional hydroxyls into drugs of the codeine series, synthesis of variously substituted naphthalenes and phenanthrenes and of various piperidine derivatives, these representing portions of the prototype morphine molecule. New derivatives of thebaine, a morphine congener, have been prepared. Other compounds which have been prepared and studied include certain aminofluorenes and some hitherto unknown isomers of cocaine.

Respiratory, Circulatory and Hematopoietic Physiology - The physiological changes in respiration and related functions have been studied in response to the stress of underwater swimming. Devices have been designed to permit variations in external respiratory resistance and in dead space. Studies with these and other instruments using trained underwater swimmers as subjects, have revealed low work efficiencies. The maximum attainable pulmonary minute volume and oxygen consumption were lower under these circumstances than for work in air. Training of swimmers resulted in either conscious disregard of the CO_2 stimulus or a lowered sensitivity of the respiratory center to CO_2. Also a conscious post-inspiratory pause, in the trained swimmer, resulted in a longer CO_2 buildup time. The maximum breathing capacity (MBC) was more sensitive toward increased inspiratory than expiratory resistance. The respiratory rate for MBC was lowered by increasing this resistance, but the tidal volume was invariant at the highest MBC for each value of resistance.

The effects of low pressures at simulated high altitudes have been further studied. A high-fat diet has been shown to increase the mortality of rats following exposure to 30,000 feet simulated high altitude, regardless of whether or not the animals are obese. Cardiovalvular thickening and the appearance of sterile valvular vegetations are more striking in obese animals. Contrary to expectations, surgically induced aortic insufficiency did not markedly interfere with tolerance of the dog to exposure to 30,000 feet simulated high altitude. At higher altitudes, this stress did increase mortality.

Insect respiration differs from that of mammals in a number of important regards. Thus it has been shown that no demonstrable oxygen debt ensues in bee-moth pupae upon short periods of anoxia. CO_2 release by diapausing saturniid moth pupae is discontinuous rather than continuous and the consequent impounding of CO_2 does not interfere with oxygen uptake.

The use of large volumes of buffered saline given orally in lieu of parenteral plasma or blood substitutes in the prophylaxis against burn shock has received complete clinical testing at Lima, Peru. 100 cases with 100 control patients have now been treated and have completely confirmed the validity of earlier impressions that this is a very effective mode of therapy. Projects related to this study include measurements of hemodynamic

quantities, electrolyte concentrations in body fluids and 17-ketosteroid excretions in the treated and control groups. Other studies relating to shock at an animal level showed that promazine and chlorpromazine protect against tourniquet shock. A number of other interesting pharmacological effects of chlorpromazine have been observed, including reduction in body temperature and decrease in swelling in the injured area.

Of the patients who survived acute shock in the Lima project, a considerable number died in the succeeding three weeks. In virtually every case death was attributed to Pseudomonas aeruginosa sepsis. Attempts to transmit this infection to mice were unsuccessful until and unless the animals were either burned or pretreated with corticosteroids. Now, with a ready source of infected laboratory animals, evaluation of modes of treatment against this infectious agent is possible and has been undertaken.

Among the plasma substitutes employed today are polyvinylpyrrolidone (PVP) and dextran. Each of these, injected into a sensitive preparation, has been shown to cause a drop in blood pressure. The mechanisms of these actions have been clarified under certain circumstances. When dextran is injected into the conscious or etherized mouse, there is no fall in blood pressure but edema occurs. In the barbiturate-treated mouse, however, a fall in blood pressure results. PVP injected into the dog causes edema and a fall in blood pressure. Either dialysis of PVP or etherization of the dog abolished the hypotension, but edema was still seen.

One of the diseases of the blood and blood-forming organs that is best understood is sickle cell anemia. This was demonstrated, some years ago, to be a "molecular" disease wherein normal hemoglobin is replaced by an abnormal hemoglobin "S". NIAMD is fortunate in having secured the services of one of the scientists responsible for this discovery. A number of additional though rarely encountered abnormal hemoglobins have been and continue to be detected, with the aid of electrophoretic techniques. Of particular interest in this regard has been the resolution of intermediate forms of ferrihemoglobins, oxidation products wherein 1, 2 or 3 of the total of four iron atoms are oxidized to the trivalent state. From the mobility differences between these products it has been possible to estimate differences in charge on normal and abnormal hemoglobin molecules.

The plasma of the anemic animal has been shown to contain an agent which stimulates erythropoiesis. This material, termed "erythropoietine" remains in the supernatant solution after alcohol precipitation of plasma at pH 4 to 5. Activity is lost after dialysis at pH 4 and the material has been shown to be thermolabile. Activity is assayed by the measurement of incorporation of radioactive iron into erythrocytes of irradiated rats. Various tags in addition to iron have been studied and

compared. Among these are radio-chromium and di-isopropylfluorophosphate-P^{32}. Comparative studies of the life span of erythrocytes have shown that in birds this expectancy is far shorter, in reptiles, far longer, than in mammals, this despite the fact that both birds and reptiles, in contrast to mammals, have nucleated red cells. The fact that mature red cells have a finite life expectancy and no capacity to replicate makes tempting the possibility of segregating the erythrocyte population according to age and studying cells of differing ages for the chemical or physical bases of aging in this simple system. Two methods of segregation, differential centrifugation and differential hemolysis, are being explored in this regard, and both appear to be promising. Studies of leucocyte metabolism, especially in relation to their reported high affinity for insulin, have been initiated.

Various Disease Processes - A variety of disease processes in experimental animals have, for one reason or another, come under scrutiny by NIAMD scientists. The desirability of securing counterparts, in small animals, of the various forms of arthritis which occur spontaneously in humans, is obvious. The comparative study of different mouse strains has revealed the presence of a genetically transmitted lesion very closely resembling osteoarthritis. This lesion was especially prominent in a congenitally obese strain. By means of diets mentioned in another section, obesity has been produced in rats which then developed osteoarthritis-like joint lesions together with adrenal changes. A strain of Streptobacillus has been encountered which is virulent in rats. After intravenous injection into young rats an infectious polyarthritis developed. At this stage organisms could be recovered from the affected joints, but not from the blood.

Among the viral diseases being studied is Rous sarcoma. The nature of the extension process has been indicated to be an invasion of the virus which transforms such normal intramuscular connective tissue as it encounters into Rous tumor cells. Newcastle Disease virus has been successfully purified by the antibody-adsorption technique described elsewhere in this report. Associated with the extensive use of monkeys in the poliomyelitis (Salk vaccine) project has been the necessity of distinguishing poliomyelitis from other similar diseases of the monkey central nervous system. Several novel diseases of this group have been encountered and tentatively classified. Another disease which has been studied is leprosy. Murine leprosy has been employed as a screening device for active antilepral agent and several drugs of promise have been encountered. Marked susceptibility to bacterial endocarditis after intravenous injection of Staphylococcus aureus and Streptococcus mitis may be produced in the dog by surgical perforation of an aortic leaflet. Diffuse proliferative glomerulonephritis often develops in the dogs receiving the latter organism.

Physical Measurements, Macromolecules - There has long been an interest and an activity at NIAMD in the effects of radiant energy upon biological systems of all sorts. The growth of such an activity is heavily dependent upon the development of novel measuring instruments and the improvement of existing equipment and this Institute has a record of contributions in this area. Notable improvements achieved in the past

year have included linearization of the wave-length coordinate of the Perkin-Elmer spectroscope-Model 13 and conversion of the transmission coordinate into the more useful optical density scale. By the application of servo methods to square-wave platinum electrode polarography, an instrument has been perfected for the high speed recording of the rate of change in oxygen concentration in solutions. Defects in the conventional sphere techniques for integrating scattered light have led to the exploration of other means of accomplishing the same end.

These improved methods have been applied to various problems of biological interest. Differences which had previously defied detection, between the infra-red spectra of lumisterol and erogsterol, have now been detected. The infra-red spectra of ATP, ADP and AMP have been compared, and whereas the first two resemble each other, the last compound is distinctly different. This difference is attributable to the presence of the pyrophosphate "energy-rich" bond in ATP and ADP, which is lacking in AMP. This attribution is based upon a detailed study of many pyrophosphates in the infra-red spectrometer. In view of the striking biochemical importance of these compounds, this new correlation of energy content and optical properties should prove of great usefulness. As model compounds for theoretical study of infra-red absorption the 12-heteropoly acid salts have served well. Tentative assignments of absorption bands to specific atom groups in these molecules have been made.

New ideas, better instruments and improved techniques have all assisted in advancing the study of photosynthesis. These studies have employed unicellular chlorophyll-containing organisms, chloroplasts derived from these and lamellate particles of chloroplasts, discovered by NIAMD scientists, which may be the ultimate photosynthesizing particles. Simultaneous measurements have been made of quantity of light absorbed at different wave-lengths, changes in oxygen concentration, and fluorescent emission, under a wide variety of circumstances. The oxygen generation in response to light has been dissected into two components, an initial "burst" which appears to be independent of the presence of CO_2, and a steady state production which requires CO_2. An immediate "gulp" of oxygen, when illumination is removed, may represent a reversal of the burst just noted. Confirmation of these suggestions awaits the completion of equipment to permit continuous recording of CO_2 concentration, currently under development. Since photosynthetic fixation of CO_2 is today recognized as merely a specific example of the more general fixation of CO_2 in which many animal cells also participate, and since energy relations, in photosynthesis, are more accessible to direct measurement, this study provides an excellent means of attacking a problem of very general biological importance.

A quite different application of spectroscopy to problems of biological interest is in the matter of hydrogen bonding. It is generally conceded today that many macromolecules such as proteins, ribonucleic acids, etc., are held together and maintained in their typical configurations by hydrogen bonds, wherein a proton in covalent linkage to one atom simultaneously occupies an unshared pair of electrons of another atom. The helical configuration of many proteins depends upon the presence of such hydrogen bridges and the denaturation of proteins is a consequence of rupture of such hydrogen bonds. In order to gain more insight into hydrogen bonds and their thermodynamic properties,

advantage has been taken of the discovery by NIAMD scientists of the facts a) that simple molecules, such as ethanol, exhibit hydrogen bonding, and b) that bonded hydrogen atoms make characteristic contributions to the infra-red spectrum. The frequencies and intensities of absorption in this spectrum have been correlated with equilibrium constants and heats of formation of hydrogen bonds. The application of nuclear magnetic resonance spectroscopy to this problem has been explored and found to be a useful tool. Hopefully it will be added to the present complement of available measurements.

Yet another novel application of spectroscopy has been in the deciphering of certain stereochemical problems in enzyme chemistry. Succinic acid-2,3-dideuterium has been prepared by two chemical methods, involving the platinum-catalyzed deuteriumation of fumaric acid on the one hand and maleic acid on the other. The two products, identical by chemical test, exhibit quite different infra-red absorption spectra and have been assigned the prefixes racemic and meso- accordingly. Interestingly, the product formed by soluble succinic dehydrogenase from fumaric acid in D_2O resembles the meso- variety, indicating that the attack by this enzyme is trans with respect to the double bond to be reduced.

The selective migration of ions across membranes has been studied both in synthetic and in biological systems. The use of permselective synthetic membranes has been extended. The anomalous behavior of certain anion-selective membranes has suggested a possible mechanism by which rapid exchange between intra- and extracellular ions might occur despite high electrical resistance between these two compartments. New membranes of special properties have been prepared using different polyelectrolytes. A theory has been developed to account for ion migration against concentration gradients. Some predictions based upon this theory have already been born out by experiments and will be further pursued. Theory and experiments have also been developed to account for the differential rates of exchange of two different species of ions across a given permselective membrane.

At a biological level the distribution of sodium and potassium across the nerve cell wall has been a matter of continued study. It has been possible, by use of drugs and metabolic inhibitors, to segregate a component of potassium flux which is dependent upon metabolic activity, active transport, from a second component of passive transport. Since cocaine affects the latter, rather than the former, its influence upon potassium influx is referred to change in membrane permeability. Elevation of the potassium concentration in the external fluid enhanced both potassium influx and outflux, the former more than the latter.

The fine structure of matter may be attacked by physical and chemical means. The use of the electron microscope is an important physical means to this end. The increasing resolution of such instruments has permitted the detection of molecular order in proteins of molecular weight of about 50,000. Direct photography of organic compounds have revealed spacings as low as 11 A in crystals of a molecular weight of about 500. This achievement represents a great increase in the power of this method. Viruses of herpes simplex and of vaccinia have been

studied in tissues and essential steps in their growth have been deciphered. The X-ray microscope, a relatively unexploited device, has developed in usefulness. The rigorous requirement of electron microscopy for ultrathin sections does not obtain here. By appropriate selection of X-ray targets to yield soft X-rays, conventional (5 micra) unstained histological sections can be satisfactorily photographed.

Chemical studies of fine structure have relied in part on such methods as ultracentrifugation, turbidimetry, osmometry, electrophoresis, diffusion, etc. In addition, methods of chemical analysis and enzyme chemistry have been invoked as needed. Comparative analyses of the muscle proteins from different species and from different muscles have shown, for example, that tropomyosins from skeletal muscle, uterus and bladder are all different. Two electrophoretically distinct tropomyosins coexist in the clam adductor. That the shortening of muscle models is not due to hydrolysis of ATP has been supported by further evidence, and the dependence of myosin adenosinetriphosphatase upon cations has been systematically studied. Incidentally this study required the development of analytical methods for ATP and ADP when present in the same solution. The structural requirements for sodium and potassium binding by myosin have been defined by a theory which is being tested. A study of the protamine salmine has been continued. This protein of fish sperm nucleoprotein has been shown to be inhomogeneous in composition. The mechanism of the fibrinogen-fibrin transformation has been further investigated, with attention directed toward the two peptides cleaved off of fibrinogen during clotting. The proteolytic nature of thrombin has been confirmed by the demonstration of C-terminal groups in these peptides and by the di-isopropylfluorophosphate inhibition of thrombin. The use of a commercial carboxypeptidase in this reaction led to the discovery of a new enzyme in this class, specific for basic amino acids. The decrease in titratable sulfhydryl groups in serum albumin on aging has been studied with respect to anion effects upon its kinetics. A mechanism for this chemical aging process in pure proteins has been suggested. It has been found possible to nitroguanylate intact proteins, converting primary amino groups into nitro-guanidino groups. Chromatographic and colorimetric behaviors of proline, hydroxyproline, 5-hydroxypipecolic and pipecolic acids have been studied. Two new amino acids, apparently related to these, have been discovered in certain fruits. γ-Guanidino butyric acid has been identified in a variety of fruits. This unusual material had previously been reported only in marine invertebrates.

A recurrent problem for the protein chemist is purification. For this reason considerable interest attaches to the development of a novel method which combines techniques of immunochemistry with those of chromatography. Appropriate adsorbents have been rendered highly specific by coating them with specific antibodies. The method permits the selective removal from a protein mixture of antigenic contaminants. It has already demonstrated its practical usefulness and may develop into a procedure of general applicability.

A form of radiant energy which will doubtless command increasing attention of biologists is the cosmic radiation found at very high

altitudes. The identification and measurements of energy and charge of heavy primary nuclei has been greatly aided by application of photo-emulsion techniques developed at NIAMD. Rockets bearing such emulsions to altitudes of 70,000 to 122,000 feet have returned with fog tracks of particles. Analysis of these tracks is yielding data of both theoretical interest and practical use in aviation medicine.

Miscellaneous Items - Histochemistry is the basis for the sciences of histoanatomy and histopathology. Staining methods, originally empirical, have over the years been progressively interpreted in terms of chemical reactions and identification of tissue reactants. New methods often evolve rationally, today, based upon knowledge of chemical reactions and of tissue constituents. To this development NIAMD scientists have made major contributions. On evidence supplied by histological studies, three previously confused pigments, melanin, trichoxanthin and neuromelanin have been distinguished from each other. From differences in their solubilities and reactivities, inferences as to their structures have been drawn. Methods of preparing and staining tissues determine in large part what may be seen under the microscope. Thus, the application of freeze-drying technique to kidney has revealed protoplasmic extensions of tubule cells into the lumina of the proximal convoluted tubules. Since this phenomenon has not been reported with other fixing methods, it becomes important to ascertain whether it represents the true anatomical state or is merely an artifact of this method of fixation. A detailed study of the reaction of hematoxylin with keratohyaline has been undertaken. Whereas the oxidized form of the dye is required, oxygen is not. Oxidized keratohyaline granules fail to combine with reduced dye. The Coons fluorescent antibody technique, which permits histological localization of antigens, has been applied to streptococcal hyaluronidase and to chorionic gonadotropin. The localization of injected gonadotropin in certain cells of the rat ovary is an important histological demonstration of an endocrinological principle.

Because potassium iodate is preferred in the tropics to potassium iodide as an antigoiter agent, it is of interest to determine its long term toxicity. In dogs, individual susceptibility to iodate was found to vary greatly. In affected animals, after three months, emesis, anorexia and weight loss were noted.

A hitherto unrecognized type of toxicity results from the treatment of foodstuffs with ethylene oxide. This volatile and reactive substance has been used in the preservation of foodstuffs. Studies at NIAMD have revealed that diets treated in this fashion are unsatisfactory for support of life and growth. This is not due to direct toxicity of ethylene oxide, since none of this reagent persists in the diet. Rather it is attributable to chemical alterations brought about by reaction between ethylene oxide and certain dietary essentials. It could be shown that the thiamine activity of an ethylene oxide-treated ration was markedly decreased.

Intramural Research Services - In addition to direct programs designed to lead to publishable research, there are a number of supporting activities which are essential to the research of others, but which do not lead directly to publication. The operation of large equipment to permit pilot plant scale isolations is such a project. By means of this equipment, several tons of horse liver have been processed for concentration of prefolic acid. Large quantities of bulbs, leaves, etc., have been processed to concentrate materials of interest to workers in NHI.

The Laboratory of Chemistry maintains a micro-analysis laboratory which this year performed about 10,000 elemental, functional group and instrumental analyses for scientists, both within and outside NIAMD. Included herein are about 1,150 infra-red spectra. Improvements in technique and the cataloging of the results of these infra-red measurements are also a part of this operation. A large number of ultraviolet spectra have also been run centrally for scientists situated in various laboratories of the institute. The mass spectrometer maintained in the Laboratory of Biochemistry and Metabolism has rendered analytical services to scientists situated in NIAMD and in NIAID. Approximately 875 individual samples have been analyzed for abundance of stable isotopes, including N^{15}, C^{13} and D^2, not counting a large number of reference and normal abundance samples. The application of scanning technique to the study of disequilibrium gases has been undertaken. A new accessory for the handling of deuterium samples has been constructed and placed in operation.

The Laboratory of Pathology maintains a centralized tissue preparation service which this year processed in excess of 53,000 slides. More than 50 different staining techniques have been employed in these preparations. Sections have been secured from about 3,700 monkeys for examination for live poliomyelitis virus as a part of the Salk vaccine program. From the Division of Indian Health and from the Bureau of Prisons materials from about 100 autopsies and 2500 surgical specimens have been prepared and studied.

During the course of the year, the Editorial Committee has processed and approved for publication about 380 manuscripts. The function of this committee is two-fold. Firstly, it scrutinizes papers for grounds for rejection. Secondly, it offers criticism to the author designed to improve the paper. In both of these capacities it has functioned admirably.

Concluding Comments - Since most of the projects described herein are continuing programs, it is not possible to come to firm conclusions in a scientific sense about them. Certain conclusions can, however, be drawn about the category of this Institute and the way it is being fulfilled by the exertions of its scientists. The distribution of efforts among the several disciplines of basic research in medical sciences is wide and appears in general to be a good and productive distribution. Collaborative ventures in research between two or more basic disciplines, between laboratory and clinic, are probably on the increase, and this, on the basis of production record, is a good thing.

One area which is regarded as of importance, and which should be strengthened, is the study of the transmission of biological information from a cell to its progeny, from a parent to its offspring. A growing interest is noted in several laboratories of NIAMD in various aspects of genetics and the inheritance both of normal and of abnormal characteristics. Since much of metabolic disease has a familial predisposition, the importance of more knowledge and understanding of the chemistry, histology and population distribution of hereditary characteristics is manifest. It is expected that future reports may contain the record of more work in this area.

A continuing problem with the scientific direction of an institute such as NIAMD is the degree to which it is profitable to try to influence the choice of problems by our scientists. Certainly the direction of the program can be influenced in the selection of new staff members to fill vacancies created either by new positions or by the departure of present staff. Also it is possible, by the distribution of support among the several laboratories, to enhance the production in an area where this seems desirable. It appears highly probable, however, that per research dollar spent, the greatest return will be secured if the mature scientist is allowed and encouraged to select the problems on which he will work. It is our belief that the meritorious and experienced investigator will in general be the wisest judge of his field of endeavor. The most important function of the Scientific Directors therefore, is in the selection of senior scientists, in their encouragement, and in the attempt to procure for them those facilities which they may require for the fulfillment of their mission.

ANNUAL REPORT

January 1 to December 31, 1956

NATIONAL INSTITUTE OF ARTHRITIS AND METABOLIC DISEASES
Clinical Investigations

Rheumatoid Arthritis

The research program in rheumatoid arthritis has been directed towards an exploration of the pathogenesis, pathology, immunological reaction, and the nature of response to a group of new, synthetic 11,17 oxygenated steroids. In conjunction with this program, studies have been done on the physiological disposition of naturally occurring adrenocortical steroids in man and the *in vitro* enzymatic metabolism of steroids.

The synovial fluid of rheumatoid arthritis is characterized by a deficiency in the polymerization of hyaluronic acid. It is believed that this may indicate a metabolic lesion in the synthesis of mucopolysaccaride by the synovial tissue. Studies on the biosynthesis of hyaluronic acid were, therefore, undertaken employing uniformly labelled C^{14} glucose, incubated with human synovial slices and, in subsequent experiments, with cell-free extract from human umbilical cord and placenta. Such a system has never been utilized before and has been found to be superior to and much more fruitful than the tissue culture technique. It has been demonstrated that synovial slices or placental cell-free extract synthesized *in vitro* hyaluronic acid and that certain of the adrenocortical and synthetic steroids inhibit the incorporation of glucose C^{14}. The effect on this synthesis of serum from patients with rheumatoid arthritis is now being studied.

A new concept of the development of morphological lesions in rheumatoid arthritis including arteritis, granulomatous synovitis and subcutaneous nodules has been advanced on the basis of intensive histopathological studies. The important role of inflammatory vascular changes in the development of these lesions has been defined. These original observations have since been widely confirmed by others. Histopathological studies have been extended in our laboratories during the past year to include vascular and other tissue changes in synovial membrane, striated muscle, integumen and viscera of other rheumatic and collagen diseases. The morphological relationship between rheumatoid arthritis, polyarteritis nodosa, systemic lupus erythematosus, systemic scleroderma and dermatomyositis was defined.

The agglutination activating factor in serum of patients with rheumatoid arthritis seems to be a protein with electrophoretic mobility in the beta globulin range. In the serum of almost all nonrheumatoid arthritics is found an inhibitory substance associated with plasma protein Fraction II of Cohn, which is probably a gamma globulin. The isolation and characterization of these serum protein components is desirable, first to enable their more effective use in diagnostic tests for rheumatoid arthritis and, moreover, to determine the role they play in the pathogenesis of the disease.

Since isolation procedures have been seriously hampered by lack of precise assay methods, the development of methods for the quantitative assay of both agglutinating and inhibitory factors have become a prime objective. It has been found that a euglobulin fraction of rheumatoid serum containing agglutinating activity also strongly inhibits hemolysis of sheep erythrocytes by Newcastle's Disease Virus and by Saponins. The method appears to have good potentialities for the quantitative assay of the agglutination activating factor. Furthermore, it has been possible to follow the rate of reaction and make some observations suggesting an optimal time of reaction for the sensitized sheep cell agglutination for best diagnostic use. The method may also be applicable to the assay of inhibitory substances. Isolation procedures can now be subjected to quantitative evaluation.

The anti-inflammatory, metabolic and pituitary-suppressing effects of new synthetic 11,17 oxygenated, 9,21 halogenated, 1,2 dehydrogenated steroids on patients with active rheumatoid arthritis have been studied. It has been observed that the potent sodium-retaining effects of 9-alpha halogenated hydrocortisone can be substantially reduced by substituting 21-desoxy for 21-hydroxyl group and can be completely overcome by substituting a fluorine atom for the hydroxyl substituent at carbon 21 even when the latter steroid was not dehydrogenated at carbons 1 and 2. Both new steroids were biologically active since they readily suppressed secretion of corticotropin by the anterior pituitary. However, these compounds lacked the anti-inflammatory potency of prednisone or 9-alpha fluorohydrocortisone. We are currently engaged in evaluation of delta-one, 9-alpha fluoro, 16-diacetate hydrocortisone. Our preliminary results indicate that this steroid possesses about the same anti-inflammatory potency as prednisone and like the latter does not cause sodium retention or potassium loss when given in conventional therapeutic

doses. In the next few months we will be the first to study a new steroid (synthesized by Josef Fried of The Squibb Institute for Medical Research), which has exhibited in the adrenalectomized rat impressively enhanced anti-inflammatory and glucocorticoid properties and has not caused retention of sodium.

Metabolism of Adrenal Steroids in Man

The physiological disposition and metabolic fate of cortisone was studied and found to be similar to hydrocortisone except that it was metabolized at a much more rapid rate. Cortisone disappeared from the plasma four times as rapidly as hydrocortisone (23 to 35 minutes), in normal subjects, and patients with liver disease. Preliminary results have been obtained that suggest that a large fraction of the cortisone is rapidly metabolized to hydrocortisone in man after oral or intravenous administration. Hydrocortisone concentration in plasma is equal to cortisone concentration within one hour after the infusion of 200 mg. of cortisone and at two hours practically no cortisone remains. Our data would seem to indicate that slightly more than one-half of the infused cortisone is metabolized to hydrocortisone. After the intra-articular injection of cortisone, it was not possible to demonstrate any significant conversion of the cortisone to hydrocortisone. After oral administration of cortisone acetate, the plasma hydrocortisone exceeds the plasma cortisone concentration by twenty or more fold.

Parallel studies on corticosterone have been done after a more accurate and reliable analytical method than existed heretofore had been developed in our laboratories employing isotope dilution. The metabolism and excretion of this naturally occurring adrenal steroid are similar to hydrocortisone; however, it is metabolized slightly more rapidly -- mean half-time, 80 minutes. Plasma concentration in normal subjects averages 1.1 μg%. Corticosterone was found to be synthesized at a rate of about 2 to 3 mg. per day, whereas the turnover rate for hydrocortisone is 17 to 29 mg. per day.

Enzymatic Studies on Steroid Substrates

Studies have been continued on elucidating new pathways by which steroids are metabolized and enzymatic mechanisms by which these metabolic changes occur. Enzymes capable of reducing various steroids have

previously been identified in this laboratory. During the past year it has been found that in the reduction of the 4-5 double bond of ring A of the steroid nucleus, a proton is attached to carbon 4 and a hydride ion from the reduced pyridine nucleotide is transferred to carbon 5 as would be predicted if the reduction did not proceed by a process of 1,4 addition and there was direct transfer of the hydrogen from the pyridine nucleotide to the substrate double bond. This is the first biochemical mechanism of its kind where a double bond is reduced directly by a pyridine nucleotide, rather than a flavoprotein.

Studies on the mechanism of steroid hydroxylation have revealed that separate systems are involved in the conversion of DOC to corticosterone and compound S to compound F. Furthermore, it has been found that two enzymes are required for each hydroxylation. These studies are of importance on several counts. First, because the adrenogenital syndrome is thought to be due to a genetic lack of one of the hydroxylating enzymes and we know, on the basis of our studies, that this is a complicated metabolic lesion. Also, since this type of reaction has its counterparts in a number of different types of compounds, an insight into the mechanism of one might be revealing for others.

Lastly, a series of related reactions in liver has been studied, and their kinetic analysis has suggested that a metabolite of TPNH, not TPN, is involved in these hydroxylations.

Gout

The research program on gout has been concerned primarily with the metabolic origin of uric acid as revealed by the pattern of incorporation of isotopically labeled precursors into urinary uric acid and urinary purines in patients with gout and hyperuricemia of various types. Clinical studies have also been made of the value of intravenous (in contrast to oral) colchicine, as a therapeutic agent, in the management of acute gout.

Although the majority of gout patients have shown a pattern of incorporation of glycine-N^{15} into urinary uric acid similar to that found in the normal, 3 patients have been found who incorporate glycine-N^{15} more promptly and to a greater extent than do normal individuals in agreement with the previous findings.

Comparison of the pattern of glycine-1-C^{14} and glycine-N^{15} incorporation into uric acid in the same gouty patient has shown an apparent anomaly. While this patient's pattern of N^{15} incorporation into uric acid in two different study periods was identical to that of the normal, his pattern of glycine-1-C^{14} was that of an "over-incorporator," despite the fact that he was excreting normal quantities of uric acid. This suggests that different portions of the glycine molecule are metabolized differently in this subject and deserves further study. It suggests that all gouty patients may be "over-incorporators" when studied with glycine-1-C^{14}.

Using another uric acid precursor, 4-amino-5-imidazolecarboxamide-4-C^{13} (AIC-C^{13}), administered simultaneously with glycine-N^{15}, it was found that one of these gouty glycine "over-incorporators" on whom studies are now complete, incorporated also more AIC-C^{13} into uric acid than did the normal. Furthermore, although in normal subjects and in gouty patients previously studied, <u>simultaneously</u> administered AIC produced a marked suppression of glycine-N^{15} incorporation into uric acid. In this "over-incorporator" AIC administration resulted in only partial suppression of glycine-N^{15} incorporation. These results suggest that this gouty patient has a defect in a regulatory mechanism for endogenous purine biosynthesis.

The pattern of glycine-1-C^{14} incorporation into urinary purines and that of AIC-C^{13} incorporation into uric acid both provided evidence for existence of two pathways contributing to uric acid formation in all subjects studied. There is a rapid direct pathway arising from newly formed purines, and a slower indirect pathway presumably arising from incorporation of the newly formed purines into tissue nucleic acids and their subsequent release with nucleic acid turnover. The extent of rapid labeling of the body urate pool in the gouty individuals after AIC-C^{13} suggests that the direct pathway to uric acid may be quantitatively more important in the gouty subjects so far studied. That the converse may be true in leukemia, with a predominance of the indirect pathway, is suggested by the increased isotope content in urinary adenine and guanine in the period 5 to 15 days after administration of glycine-1-C^{14}.

Indirect evidence suggesting that a pathway for urate synthesis other than that involving xanthine oxidase may be present in the human has been deduced

from the time course of appearance of isotope in urinary oxypurines and uric acid after feeding glycine-1-C^{14}. Maximal labeling of uric acid is achieved on the second or third day with isotope concentrations substantially above that of the oxypurines at that time. The curves do not indicate a direct precursor-product relationship and are consistent with the interpretation that most of the uric acid formed arises from precursor pools different from those sampled by the urinary purines.

Further evidence for a pathway of urate synthesis that does not involve xanthine oxidase is provided by the discovery of a new enzyme in beef liver that produces uric acid from uric acid riboside. This enzyme is being purified and characterized, and the detailed steps of this metabolic pathway are under investigation.

Treatment of acute gout with intravenous colchicine is at times superior to the oral route of administration. In treating 20 acute attacks, no serious toxic effects were found, and in most cases relief was more prompt and fewer gastrointestinal symptoms occurred.

The Physiology of Diseases of the Thyroid

Research on the thyroid has been directed towards an elucidation of the pathogenesis of the changes that occur in congenital athyreosis (cretinism), and towards the biochemical defects in the formation of thyroid hormone which result in the development of goiters. In addition, the mechanism of action of thyroid hormone on an enzymatic level and on a physio-chemical level has been the subject of considerable work.

A group of individuals with congenital athyreosis has been studied extensively over a 12 month period, during which the responses to treatment were followed. Psychological testing has shown a rather unusual intra-individual consistency of development. Any one individual appears to have been retarded to about the same level in all facets of intelligence and development. Electroencephalogram studies have shown absent alpha waves and general dysrhythmia. Interpretations have been difficult because of the paucity of control data on the normal children. It is interesting that partial response in the EEG has been seen as early as 12 hours after the administration of triiodothyronine. The pattern of maturation of bone has been studied and the influence of thyroid hormone has been noted, but

malnutrition without thyroid abnormality has been shown to result in similarly delayed bone maturation. It is hoped to elucidate the separate roles of growth hormone, thyroxine, androgen, and nutrition on bone maturation. The effect of treatment with very large doses of thyroxine, triiodothyronine and triiodothyroacetic acid has been studied. No particular advantage accrues to any one of these agents. The toxicology of triiodothyronine in children has been delineated. Hypertension has been noted (2 of 9 cretins, 0 of 18 normals) and spider angiomata have been seen without evidence of liver disease (0 of 9 cretins, 4 of 18 normals).

Biochemical Abnormalities in the Formation of Thyroid Hormone as a Cause of Goiters

On the assumption that iodine deficiency and naturally occurring goitrogens play a minor role in the pathogenesis of most cases of goiters in this area, a systematic study of patients with goiters has been initiated. One goitrous cretin has been studied who showed a congenital absence of the enzyme responsible for the first step in the iodination of tyrosine, although the ability to concentrate iodine was preserved. Although cases such as this are a statistically insignificant cause of cretinism, they are of considerable physiological interest, because they permit the study of the iodide concentrating mechanisms completely separated from organification. The effect of changes in the level of TSH in this system was studied. It was shown that exogenous TSH caused no change in iodide concentration when the level of TSH was 1.0 milli-unit/ml and reduction of the level of TSH to less than 0.4 of a milli-unit/ml by administration of triiodothyronine also was without effect. There has been considerable discussion as to whether antithyroid drugs of the thiouracil types increase iodide concentration by the thyroid. This has been studied in the patient mentioned above and no effect has been noted.

A further study of euthyroid adults with goiters has been pursued by an *in vitro* approach. After surgical removal of the goiters, they have been incubated with I^{131} and the method has been developed so that this tissue will synthesize thyroglobulin from I^{131} (or, as in one case, tyrosine C_2^{14}). The iodinated compounds after hydrolysis of thyroglobulin show no significant difference between normal and goitrous glands. However, 3 previously unknown iodinated compounds have been found

and their structure is currently under investigation. The physical properties of thyroglobulin studied by electrophoresis and salting out, have shown no significant difference between normal and goitrous glands. In one individual with carcinoma of the thyroid, however, an iodinated protein was found which was unusually soluble in phosphate buffer and which had a different electrophoretic mobility from normal thyroglobulin. No reason for the development of goiters in these patients has yet been found.

Mechanism of Action of Thyroid Hormone

Since thyroxine is firmly bound to plasma proteins, a study has been conducted to determine the concentration of free or unbound thyroxine in blood and other biological fluids. A method termed "Reverse Flow Electrophoresis" has been developed so that the concentration of sites on the particular protein (TBP) in serum which binds thyroxine can be determined. Equations have been developed so that the level of free thyroxine in serum may be calculated. The level of free thyroxine was found to be 6 x 10^{-11}M. This level was variable in nephrosis, but essentially normal. In hypothyroidism, there was marked depression and in hyperthyroidism marked elevation of free thyroxine in serum. It was also shown that the amount of thyroxine binding protein was elevated in pregnancy or during treatment with estrogens. From these and other data it has been suggested that the physiological activity of thyroid hormone is proportional to the level of free thyroxine in serum, and that the rate of degradation of thyroxine is likewise proportional to the concentration of free thyroxine in serum.

Studies have also been conducted on the effect of a variety of thyroactive compounds on isolated enzyme systems. It has been shown that mammalian malic and glutamic dehydrogenase are inhibited by 10^{-5}M thyroxine and triiodothyronine. Dinitrophenol has no effect on these enzymes, although highly halogenated phenols and xanthine dyes are also active. In inhibiting these enzymes, the activity of these compounds was roughly proportional to the degree of halogens plus a decreasing order of activity of the halogens in the series of $I > Br > Cl$. A number of other pyridine nucleotide linked dehydrogenases were shown to be inhibited by thyroxine but to a lesser extent. Several proteinases were not effected as measured by pseudo esterase activity.

Whether the effects of thyroid hormones on dehydrogenases is of major importance in the ultimate action of thyroxine is not known, but these are the first studies to reveal *in vitro* effects of thyroxine in isolated enzyme systems and, as such, represent a significant advance.

Diabetes

Research in diabetes has been directed towards oral antidiabetic drugs, vitamin metabolism in diabetes, the effect of insulin on pentose metabolism, and galactose diabetes. Carbutamide (BZ-55) has been found to be active in certain cases of diabetes and not effective in others, in terms of lowering the blood sugar and the urinary loss of sugar. However, the first case of death from BZ-55 was noted and reported. On the basis of this and subsequent similar reports by others, this drug was withdrawn from use.

The metabolism of thiamine, riboflavin, pantothenic acid, nicotinic acid, and vitamin B_{12} have been studied in normal individuals, in diabetics with and without complications. It has been shown that both types excrete in the urine a greater than normal amount of a test dose of thiamine. Changes in riboflavin and pantothenic acid are inconclusive. There were no changes in diabetes in the metabolism of vitamin B_{12}. These data are difficult to interpret but suggest that there may be a defect in diabetes in the ability to utilize thiamine.

The metabolism of a variety of pentoses has been investigated both as a matter of intrinsic interest and as a means of verifying in man the concept that insulin acts by increasing the diffusion space for glucose. In general, the results confirm and extend previous observations: for example, D-xylose and L-arabinose were responsive to the effect of insulin whereas D-arabinose and D-lyxose were insensitive. D-ribose is of considerable interest since it seems to cause a decrease of as much as 60% in the blood glucose. Studies with C^{14} labeled D-ribose showed substantial degradation to CO_2 and by comparison of the C^{14} activity in urine with ribose content, the presence of several nonribose metabolites was demonstrated. Identification of these metabolites is being pursued. Insulin was shown gradually to increase the rate of loss of D-ribose from blood. These studies confirm in man the effect of insulin on penetration of sugars of a certain type into cells and furthermore suggest the possible importance of certain pentoses in carbohydrate metabolism.

Enzymatic Defect in Galactosemia

A specific enzymatic defect was found in the blood and liver cells of patients with this disorder. The apparently hereditary absence of the enzyme P-gal-transferase results in a block in the conversion of galactose 1-phosphate to glucose 1-phosphate and accounts for the inability of children with disease to utilize milk sugar. A specific spectrophotometric method for blood has been developed by which the disease can be accurately diagnosed. With this diagnosis made and the intake of milk avoided, babies with this disorder may now be expected to grow normally and avoid the mental and visual defects which, up to now, have been a serious consequence of galactosemia.

Determination of Basis for "Direct" and "Indirect" Bilirubin Reaction

Study of the metabolism of bile pigments appears to have solved a problem which has been under discussion for some 40 years. The factors responsible for the difference between "direct" and "indirect" reacting bilirubin pigments have not been clearly understood. It has now been shown by original work in our laboratory that direct-reacting bilirubin is water-soluble bilirubin glucuronide and the indirect form is free bilirubin. The process has now been considerably clarified by which bile pigments are converted by liver cells into a form suitable for excretion into the bile ducts. This work has special significance for the disease congenital hyperbilirubinemia (true Gilbert's disease) in which it is beginning to appear that such patients have a profound defect in hepatic ability to conjugate metabolites with glucuronic acid.

Discovery of New Type of Congenital Hemolytic Anemia

A previously unknown and unusual form of congenital hemolytic anemia was discovered by NIAMD hematologists in a father and son afflicted by life-long severe anemia with jaundice and splenomegaly. In the peripheral blood, of the father and son, reticulocytes and mature erythrocytes were found to contain one and occasionally two round or oval-shaped inclusion bodies. These unusual particles were demonstrated by phase microscopy and by

supravital staining with aqueous methyl violet, were usually seen in the center of the cell and were found to remain in the cell stroma after hemolysis.

In both patients with the rare hemolytic anemia, the urine was found to contain a rare dark brown pigment which, on chemical characterization, appeared to belong to the mesobilifuchsin group, a type of pigment never before isolated from urine. After transfusion of the abnormal cells to the normal recipient, the latter excreted the same pigment. This indicated that the anemic cells in this disorder have a defective mode of breakdown leading to mesobilifuchsin formation rather than a normal breakdown to bilirubin.

New Method for Localizing Obscure Site of, and Measuring Blood Loss from Gastrointestinal Tract

Use of the Cantor tube with periodic aspiration of intestinal contents as the tube is passed down the intestinal tract, in conjunction with chromate-tagging of the patient's red cells for aid in the detection of blood in the intestinal contents, provides a valuable tool to clinicians in the search for sites of intestinal blood loss. The value of this procedure was proven in five instances (including one malignancy) in which sources of blood loss were previously missed by standard methods, including radiographic examination and life-saving surgery was performed.

Effective Chelating Agent for Copper in Wilson's Disease

Preliminary studies with a preparation of penicillamine have demonstrated that this is a very effective compound for removing copper in Wilson's disease. This compound, given orally in doses of 4 grams daily for 3 days will produce a 10- to 20-fold increased excretion of copper in the urine, and has an advantage in that it can be given orally. It causes no increased excretion of iron in hemochromatosis.